EXHIBIT 2

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Identification of Murine Protective Epitopes on the *Porphyromonas gingivalis* Fimbrillin Molecule

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Fimbriae from *Porphyromonas gingivalis* are believed to play an important role in the pathogenesis of periodontal diseases. The aim of the present study was to identify the fimbrial protective T-cell epitopes in CBA/J mice. A truncated protein corresponding to amino acids 1 to 198, PgF1-198, was generated and allowed us to demonstrate that the N terminus of the protein contains T-cell epitopes. With synthetic peptides, an immunodominant sequence was identified between amino acids 103 and 122. The corresponding peptide, PgF-P8, induced T-cell proliferation after in vitro restimulation of in vivo-primed cells, giving a stimulation index comparable to the one obtained with r-fimbrillin, and induced production of both Th1 and Th2 cytokines. Growth supernatant contained significant levels of interleukin 2 (IL-2), gamma interferon, IL-4 (28 pg/ml), and tumor necrosis factor alpha. Immunization of mice with r-fimbrillin, PgF1-198, and PgF-P8 induced production of antibodies specific to r-fimbrillin and PgF-P8. In addition, by using the mouse chamber model we found that mice immunized with PgF-P8 were dramatically protected against a normally lethal injection of *P. gingivalis*. Animals immunized with PgF-P8 40 days prior to challenge showed a 60% survival rate when challenged with *P. gingivalis*, compared with just 25% survival in control animals and just 5% survival in mice immunized with PgF-P8 only 21 days prior to challenge. Although the protection depended on the time of immunization before the bacterial challenge, it did not correlate with in vivo local cytokine production (IL-2, IL-4, IL-6, tumor necrosis factor alpha, and gamma interferon), specific antibody levels, or the isotype of anti-PgF-P8 antibodies produced.

Porphyromonas gingivalis fimbriae are believed to play an important role in the pathogenesis of periodontal diseases. Inactivation of the fimbrial gene *fimA* was shown to diminish periodontal bone loss in gnotobiotic rats (14). Colonization of the subgingival area by *P. gingivalis* is likely mediated by fimbriae through their affinity for human crevicular cells (9), human buccal epithelial cells (11), salivary proteins (12), and preexisting oral microflora (8). In addition to their role in adhesion, they are also known to stimulate both humoral and cellular immune responses of the host. Patients with periodontal diseases have elevated serum antibody titers to *P. gingivalis* fimbriae (16, 27) and to synthetic peptides derived from their sequences (15). Induction of various proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), IL-6, and IL-8 in vitro by *P. gingivalis* fimbrillin or synthetic peptides based on its primary amino acid sequence has also been reported (17, 18, 20).

Immunization with fimbriae or corresponding synthetic peptides protects against subsequent *P. gingivalis* infections. In the rat, immunization with purified fimbriae prevents periodontal destruction induced by *P. gingivalis* infection (3). Immunization of guinea pigs with a synthetic peptide corresponding to amino acids (aa) 202 to 221 of the native fimbrial subunit, the fimbrillin, protected them from cutaneous lesions caused by *P. gingivalis* local injections (15). *P. gingivalis* fimbrillin is a potent polyclonal activator of B cells (17) and was recently shown to be T cell reactive as well (15, 21). It still remains to be determined if this protection is mediated predominantly by B-cell and/or T-cell activity.

The aim of this study was to identify T-cell immunodominant regions of the fimbrillin molecule and determine if they contain protective epitopes. The presence of murine Th1 and Th2 epitopes on the fimbrillin molecule was recently reported (21). One synthetic peptide capable of inducing both Th1 and Th2 cytokines also induced major histocompatibility complex-restricted delayed-type hypersensitivity in mice (21). The different cytokines produced by the two T-helper subsets can have contrasting effects on the evolution of an immune response. The roles of Th1 and Th2 cells in the progression or prevention of a pathologic state have been reported in the case of diabetes (22), leprosy (13), and helminth infections (4), among others.

In the present report, we found that the N-terminal portion of the fimbrillin molecule contains T-cell epitopes. With synthetic peptides covering the N-terminal sequence, an immunodominant region was located between aa 103 and 122. The corresponding peptide, PgF-P8, induced specific antibody production in immunized mice and in vitro production of both Th1 and Th2 cytokines and also conferred protection against *P. gingivalis* infection in the mouse chamber model.

MATERIALS AND METHODS

Animals. Female CBA/J mice 6 to 8 weeks old were purchased from Jackson Laboratories (Bar Harbor, Maine).

Bacteria. For the infection experiments, *P. gingivalis* ATCC 33277 was grown in Wilkins-Chalgren broth (Unipath Ltd., Basingstoke, England) under anaerobic conditions for 48 h. Cells were harvested and resuspended in prereduced Wilkins-Chalgren broth to a concentration of 3.75×10^{11} CFU/ml, assuming that 10^9 CFU/ml gives an optical density at 660 nm of 1.2 (6).

Recombinant proteins. *P. gingivalis* r-fimbrillin was purified from *Escherichia coli* clone OW.2 as already described (26). Another r-protein, a truncated form of the native fimbrillin, was expressed in an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible type III expression vector from the QIAexpress system (Qiagen, Chatsworth, Calif.). This type of construct includes a 6-histidine affinity tag at the C terminus of the protein and uses the 5′ start and 3′ stop codons of the vector. The 6-His tag is poorly immunogenic and allows easy purification of the

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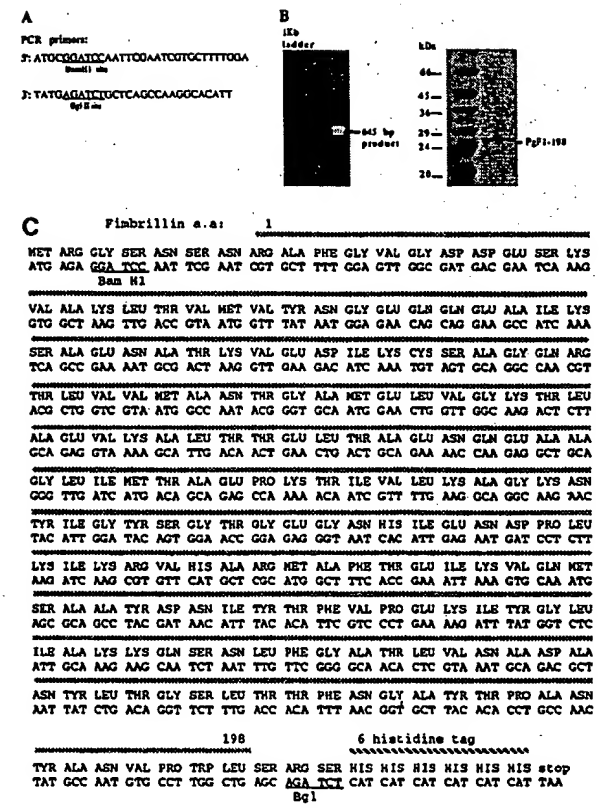

FIG. 1. (A) Set of PCR primers used to prepare the insert containing the N-terminal portion of *P. gingivalis* fimbrillin. A *Bam*HI site and a *Bgl*II site were included in the 5' and 3' primers, respectively, in order to allow cloning into the PQE system of vectors. (B) Left side, PCR product obtained with the above set of primers and the plasmid OW.2 as a template. The reaction was run for 40 cycles of 92°C for 30 s, 57°C for 30 s, a ramp of 1°C for 4 s up to 72°C, and then extension for 30 s. Right side, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the r-protein. PgF1-198 was expressed after cloning of the PCR product into PQE-50/16 vector and transformation of *E. coli* M15 cells. The transformant PQE600.9 was induced with IPTG, and the r-protein was purified on a Ni-NTA column under denaturing conditions (see Materials and Methods). The gel was stained with Coomassie blue. (C) Sequence of the plasmid isolated from the clone PQE600.9. The sequence corresponds to an N-terminal portion of the *P. gingivalis* fimbrillin gene, with some extra amino acids at the C and N termini as a consequence of cloning into the PQE system.

protein on immobilized Ni^{2+}, the Ni-nitrilotriacetic acid (NTA) resin (Qiagen). The truncated gene, including the coding sequence for aa 1 to 198 of the native form of fimbrillin, was obtained by PCR with OW.2 vector as a template. The primers were designed to include a *Bam*HI restriction site at the 5' end and a *Bgl*II site at the 3' end to allow ligation into the type III PQE-50/16 vector. The sequences of the primers are given in Fig. 1A. *E. coli* M15 cells were transformed with the construct according to the manufacturer's instructions. Clones were screened for the expression of a 23-kDa protein binding to Ni-NTA agarose after induction with IPTG. One clone, PQE600.9, was selected, and the r-protein, PgF1-198, was purified on a Ni-NTA column under denaturing conditions in 8 M urea, according to the manufacturer's instructions. Refolding of the protein was accomplished by stepwise dilution of the urea. First, the denatured protein was added drop by drop to 10× volumes of 1 M urea–0.05 M Tris-HCl–0.005% Tween 80–2 mM reduced glutathione–0.02 mM oxidized glutathione, pH 9.6, while stirring. After dialysis in 0.05 M Tris-HCl, pH 9.6, to remove the remaining urea, the refolded protein was concentrated in an ultrafiltration stirred cell (Model 8050; Amicon, Beverly, Mass.), and any insoluble aggregated protein was removed by centrifugation. Sequence of the PQE600.9 construct was verified by sequencing at the University of North Carolina at Chapel Hill Automated Sequencing Facility on a Model 373 DNA sequencer (Applied Biosystems) with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Synthetic peptides. Synthetic peptides, based on the amino acid sequence of the native fimbrillin of *P. gingivalis*, were synthesized as 20-mers overlapping on 5 aa covering aa 1 to 196 at the University of North Carolina PMBB Micro Protein Chemistry Facility with a multiple peptide synthesizer (Rainin Symphony). Peptides were purified by reverse high-performance liquid chromatography and analyzed by mass spectroscopy. For immunizations, peptides were conjugated to bovine serum albumin (BSA) and mixed with complete Freund's adjuvant. For in vitro restimulations, free peptides were diluted in RPMI medium.

Proliferation assay. CBA/J mice were immunized in the footpads with 20 μg of either r-fimbrillin or PgF1-198 per animal. Seven days later, popliteal lymph nodes were removed and single-cell suspensions were obtained. B cells were removed by passage of the suspension through an anti-immunoglobulin (Ig) column (Cellect Mouse T column; Biotex Laboratories Inc., Edmonton, Canada). Cells were then cultured in 96-well plates (Costar) at a concentration of 2×10^5 cells per well in the presence of r-proteins or peptides in RPMI 1640 supplemented with 10% fetal calf serum (Cellect Silver; Flow Laboratories). After 3 days, 1 μci of [^{3}H]thymidine (60 to 90 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was added to each well, and the cells were cultured for another 3 h before being harvested on glass fiber filters. The incorporated radioactivity was measured with a liquid scintillation counter.

Chamber infections. Coil-shaped subcutaneous chambers were surgically implanted in the subcutaneous tissue of the dorsolumbar region of each mouse (7). For protection experiments, mice were immunized with PgF-P8–BSA or BSA, 20 μg per animal, in complete Freund's adjuvant in the footpads, at the time of implantation. This immunization method was approved by the Institutional Animal Care and Use Committee, Division of Laboratory Animal Medicine, University of North Carolina. At 21 to 40 days postimmunization, the mice were challenged by injection of 0.1 ml of *P. gingivalis* ATCC 33277 (3.75×10^{10} CFU) into chambers. Mice were examined daily for health status. Chamber fluids were collected with a 26-gauge hypodermic needle and syringe, 1 to 3 days before challenge and daily after challenge. Immediately after sampling, the chamber fluids were diluted into 1 ml of phosphate-buffered saline (PBS) containing 2% BSA and cleared of bacteria by centrifugation. Serum was obtained from blood collected retro-orbitally, prior to immunization and periodically after immunization.

Cytokine enzyme-linked immunosorbent assay (ELISA). T-cell culture supernatant and chamber fluid cytokine levels (IL-2, IL-4, IL-6, gamma interferon [IFN-γ], and TNF-α) were measured with minikits from Endogen (Cambridge, Mass.). The assays were performed according to the manufacturer's instructions.

Antibody ELISA. Antibodies reactive with PgF-P8 were measured in both sera and chamber fluids by ELISA. Briefly, 96-well plates were coated with r-fimbrillin (10 μg/ml) or PgF-P8 (5 μg/ml) in carbonate buffer (pH 9.6) for 75 min at 37°C. Unoccupied sites were blocked with PBS containing 2% BSA and incubated overnight at 4°C. After washing of the plates three times with washing buffer (50 mM Tris, 0.5% Tween 20), the samples, diluted in PBS–2% BSA, were incubated 1 h at 37°C. After three washes, an anti-mouse IgG, IgA, and IgM conjugated to biotin (Zymed, South San Francisco, Calif.) diluted 1:5,000, were incubated for 1 h at 37°C. After three more washes, streptavidin conjugated to horseradish peroxidase (Zymed) diluted 1:8,000 was incubated 30 min at 37°C. After three final washes, the plate was developed with TMB (Dako Corporation, Carpinteria, Calif.) for 30 min in the dark, the reaction was stopped with 0.18 M H_2SO_4, and the results were read at 450 nm (with 540 nm as a reference) on an automated ELISA plate reader (Bio-Rad Laboratories). Optical density values were multiplied by the dilution factor and expressed as ELISA units (EU).

Statistical analysis. Comparisons of specific antibody and cytokine levels were made with Student's *t* test.

RESULTS

Cloning and expression of the fimbrillin truncated gene. An insert corresponding to an N-terminal portion of the whole fimbrillin gene was obtained by PCR (Fig. 1B, left panel). After ligation into a type III PQE expression vector and transformation into *E. coli* M15 cells, one clone, PQE600.9, was selected for its expression of a 23-kDa protein binding to Ni-NTA after induction with IPTG (Fig. 1B, right panel). The sequence of the insert was obtained and confirmed its relation to the native fimbrillin (Fig. 1C). The protein was purified on an Ni-NTA column under denaturing conditions, refolded properly, and used as the antigen in our experiments.

T-cell response to r-fimbrillin and PgF1-198. The presence of T-cell epitopes on the truncated fimbrillin was verified by immunizing mice with either form of the protein and then restimulating in vitro 7 days later. This restimulation, with either r-fimbrillin or PgF1-198, induced T-cell proliferation as shown in Fig. 2. Although the stimulation indices (SI) were slightly lower for restimulation with the truncated protein, with

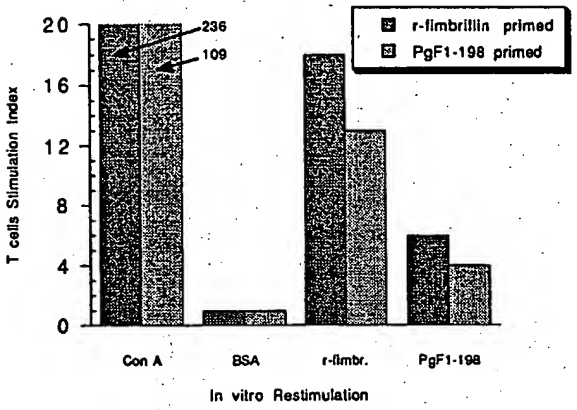

FIG. 2. T-cell response to r-fimbrillin and to PgF1-198. CBA/J mice were immunized in the footpads with 20 μg of either antigen per animal in complete Freund's adjuvant. Seven days later, the lymph node T cells were restimulated in vitro for 72 h. [^{3}H]thymidine was added to the culture for the last 3 h, the cells were harvested, and the incorporated radioactivity was measured. Results are expressed as SI = incorporated radioactivity in the activated cells divided by incorporated radioactivity of the nonactivated control cells. Con A, concanavalin A.

an SI of 6 compared with an SI of 18 for the whole protein, they were significant compared with BSA (SI = 1).

Localization of the T-cell epitopes. We tried to further localize the potential epitopes on this portion of the molecule by testing a panel of 20-aa peptides, overlapping on 5 aa, for their capacity to induce a proliferative response to r-fimbrillin-primed T cells. CBA/J mice were immunized with r-fimbrillin in the footpads, and 7 days later, lymph node T cells were subjected to in vitro restimulation. Among the peptides tested, only one generated a proliferative response when mixed with T cells. All the other peptides did not give a significant proliferative response compared with the one induced by the whole

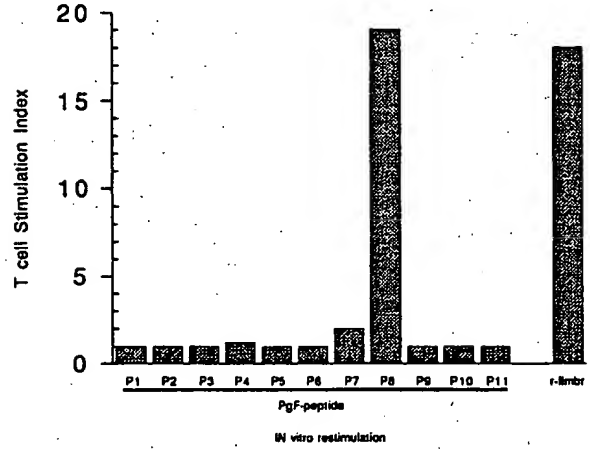

FIG. 3. T-cell responses to synthetic peptides corresponding to *P. gingivalis* fimbrillin N-terminal sequence. CBA/J mice were immunized in the footpads with 20 μg of r-fimbrillin per animal in complete Freund's adjuvant. Seven days later, the lymph node T cells were restimulated in vitro with the peptides, for 72 h. [^{3}H]thymidine was added to the culture for the last 3 h, the cells were harvested, and the incorporated radioactivity was measured. Results are expressed as SI = incorporated radioactivity in the activated cells divided by incorporated radioactivity of the nonactivated control cells.

TABLE 1. Cytokine production by r-fimbrillin-primed T cells after in vitro restimulation

Restimulation	Cytokine (pg/ml)			
	IL-2	IFN-γ	IL-4	TNF-α
None	483	3,902	0	<80
r-Fimbrillin	757	>93,000	0.2	<80
PgF-P8	6,202	>93,000	28.0	<80

molecule (Fig. 3). The reactive peptide, PgF-P8, corresponds to aa 103 to 122 of the native *P. gingivalis* fimbrillin protein. Lymph node T cells primed with r-fimbrillin responded in vitro to 5 μg of r-fimbrillin with an SI of 18; an equivalent amount of PgF-P8 gave an SI of 19 (Fig. 3). Similar results were obtained with T cells primed with PgF1-198, i.e., an SI of 13 when restimulated with r-fimbrillin and an SI of 18 when restimulated with 5 μg of PgF-P8 (data not shown).

Cytokine synthesis pattern of the PgF-P8-reactive T cells. The cytokines produced upon in vitro restimulation of r-fimbrillin-primed T cells with r-fimbrillin or PgF-P8 were analyzed by ELISA. Both r-fimbrillin and PgF-P8-reactive T cells had elevated levels of IL-2 in their growth supernatant compared with nonstimulated cells, although PgF-P8 induced a much higher concentration, 6,202 pg/ml, compared with 757 pg/ml for r-fimbrillin (Table 1). IFN-γ was secreted by PgF-P8 and r-fimbrillin-reactive T cells at a very high concentration, >93,000 pg/ml, whereas nonstimulated cells had a level of only 3,902 pg/ml. IL-4 was not detected in nonstimulated cell supernatant and was present at very low levels in r-fimbrillin-restimulated cell supernatant (0.2 pg/ml) but was present at higher levels in supernatant from PgF-P8-restimulated cells (28 pg/ml). TNF-α was present only at very low levels (<80 pg/ml) in supernatant from nonstimulated cells and r-fimbrillin- or PgF-P8-stimulated cells.

Humoral response to PgF-P8. CBA/J mice were immunized with r-fimbrillin, PgF1-198, or PgF-P8 in footpads. Nineteen days later, levels of antibodies against PgF-P8 in the serum were determined by ELISA. Compared with preimmune serum, all immunized animals had increased levels of serum antibodies to PgF-P8 although levels varied greatly between individual animals. PgF-P8-immunized mice had the highest levels with an average of 561 ± 328 EU; PgF1-198 induced levels of 188 ± 114 EU. Animals immunized with r-fimbrillin produced a significant but modest amount of specific antibodies with 44 ± 10 EU, and these levels were only slightly higher than the naive levels (21 ± 12 EU).

Protective immunization with PgF-P8. We then used the mouse chamber model to determine if PgF-P8 could confer any protection against *P. gingivalis* infection. CBA/J mice were immunized, in the footpads, with PgF-P8 conjugated to BSA at the time of implantation of the subcutaneous chamber. The animals were divided into two groups; one group (n = 17) was challenged with 3.75×10^{10} CFU of *P. gingivalis* ATCC 33277 21 to 27 days after the immunization. The other group (n = 10) was challenged with the same inoculum 40 days after the immunization. BSA-immunized animals were included as controls in each group, as were naive animals.

For analysis purposes, postchallenge day intervals (1 to 3 days, 4 to 7 days, and 8 to 14 days) were used to regroup infection outcomes. Of the nonimmunized control group, 15 of 20 (75%) died within 7 days of the challenge (Table 2). Three animals (15%) rejected the chamber, usually between 7 and 14 days postchallenge (one animal rejected the chamber the day following the challenge). Two mice (10%) maintained the

TABLE 2. Protective effect of PgF-P8 in *P. gingivalis* mouse chamber infection

Group	No. of deaths per period of days[a] (%)			No. of survivals at 14 days[a] (%)
	0 to 3 days[b]	4 to 7 days	8 to 14 days	
Control	7/20 (35)	8/20 (40)	0/20 (0)	5/20 (25)
BSA (21 days)	2/2 (100)	0/2 (0)	0/2 (0)	0/2 (0)
BSA (40 days)	5/6 (83)	0/6 (0)	0/6 (0)	1/8 (17)
PgF-P8 (21 to 27 days)	16/17 (94)	0/17 (0)	0/17 (0)	1/17 (6)
PgF-P8 (40 days)	2/10 (20)	2/10 (20)	0/10 (0)	6/10 (60)

[a] Ratio of number of deaths to total number of mice.
[b] Days after challenge with 3×10^{10} CFU of *P. gingivalis* ATCC 33277.

chamber for at least 14 days. Of the BSA-immunized animals in both the 21- to 27-day and the 40-day groups, 88% (seven of eight) died within 7 days of the challenge. The last animal survived and maintained the chamber for at least 14 days.

The results we found with the PgF-P8 animals are somewhat surprising. There was a dramatic protection difference depending on how long after immunization we waited before challenging the mice with *P. gingivalis* cells. The group of animals challenged 21 to 27 days after immunization with PgF-P8 followed a similar pattern as the control groups. A total of 16 of the 17 (94%) animals died within 3 days. Only one animal (6%) survived and maintained the chamber over 14 days. In the group of animals challenged 40 days after immunization with PgF-P8, we observed a dramatic shift in the death rate. Only 2 of 10 (20%) died within 3 days followed by another 2 of 10 (20%) between days 4 and 7 (actually, both on day 6) for a total of only 40% death in that group.

The survival rate of this latter group was the highest, i.e., 60% compared with 25% for the naive, 13% for both BSA-immunized groups, and 6% for the group of mice PgF-P8 immunized 21 to 27 days prior to challenge. Table 2 compiles death and survival data for all five groups.

Antibodies specific to PgF-P8 in serum and chamber fluid prior to challenge. We sought to determine which immune parameter might be responsible for protection in PgF-P8-immunized animals by measuring specific antibodies and immune cytokine production in *P. gingivalis*-challenged animals. Antibodies specific for PgF-P8 were assayed by ELISA in the serum and the chamber fluid of the mice immunized with this peptide prior to challenge with *P. gingivalis* cells. There was an elevation of about 10 times the baseline level of serum specific antibodies 14 days after the immunization, in both animals challenged after 21 to 27 days and those challenged after 40 days (Table 3). These specific antibodies were also present in the chamber, at a lower concentration, as tested 3 days prior to challenge for both groups. It corresponded to 18 to 24 days after immunization for the animals challenged after 21 to 27 days and to 37 days after immunization for those challenged after 40 days. Surprisingly, the highest antibody concentrations were observed in the group of animals that had been immunized for the longer time, although the difference between the averages of the two groups was not significant ($P > 0.05$). There was no correlation between the individual antibody levels in the serum or in the chamber and the outcome, i.e., death or survival ($P > 0.05$). Nonimmunized and BSA-immunized animals had no or very few antibodies to PgF-P8 in both their serum and their chamber fluid (data not shown).

Cytokines present in the chamber after challenge. We first analyzed the immediate cytokine response in mice challenged with *P. gingivalis* cells. Chamber fluids were collected 1 h after the injection of bacterial cells and tested for the presence of IFN-γ, TNF-α, IL-2, IL-4, and IL-6. For most cytokines tested, there was no significant difference between level of production and immunization. A detailed analysis of some of the cytokines tested in representative individuals of each group of animals is given in Table 4. There was no significant difference between IL-4 production and any immunization pattern ($P > 0.05$); the same was true for IL-2 and IL-6. The mean level of IFN-γ was significantly lower for the group of animals immunized with PgF-P8 40 days prior to challenge compared with animals immunized 21 days prior to challenge ($P < 0.05$). For all the other groups, there was no significant difference in IFN-γ levels ($P > 0.05$). TNF-α levels were significantly higher in the group of animals immunized with PgF-P8 40 days prior to challenge compared with all other groups ($P < 0.05$). Interestingly, this group of animals is the one showing the highest survival rate. TNF-α levels were not significantly different by comparison of the other groups of animals with each other ($P > 0.05$). Furthermore, in examining individual mice, there was no correlation between any cytokine level and the outcome of the infection, including IFN-γ and TNF-α ($P > 0.05$).

Since most deaths occurred later than 48 h after challenge and levels at 1 h did not seem to correlate, we then verified whether different kinetics secretion of certain cytokines over several days would be associated with death versus survival. For this purpose, chamber fluid was sampled daily after challenge with *P. gingivalis* cells and again tested for the presence of cytokines. IFN-γ, IL-4, and TNF-α kinetic curves of representative individuals are shown in Fig. 4. Generally, we ob-

TABLE 3. Amount of serum and chamber antibodies to anti-PgF-P8 and outcome of *P. gingivalis* infection

Immunization profile	Serum, preimmunization[a]	Serum, 14 days after immunization[a]	Chamber, 3 days prior to challenge[a]	Outcome, 14 days after challenge
PgF-P8, 21 days	19	224	31	Died
	17	96	7	Died
	17	643	89	Died
	30	72	10	Died
	11	141	25	Died
	19	ND	13	Died
	21	265	33	Died
	49	ND	26	Died
	15	78	6	Died
	2	ND	8	Died
Mean ± SD	20 ± 12	217 ± 202	25 ± 25	
PgF-P8, 40 days	17	74	13	Died
	32	284	83	Survived
	27	356	127	Died
	19	249	97	Died
	25	125	8	Died
	44	ND	1	Survived
Mean ± SD	27 ± 10	217 ± 116	55 ± 54	
BSA, 21 days	18	ND	1	Died
	2	3	2	Died
Mean ± SD	10 ± 11	3 ± 0	1 ± 0.2	
BSA, 40 days	39	37	2	Died
	29	36	4	Died
	23	ND	11	Survived
	13	1	3	Died
	2	ND	11	Died
Mean ± SD	21 ± 14	24 ± 20	6 ± 5	

[a] Antibody levels expressed as EU. ND, not done.

TABLE 4. Chamber fluid cytokine levels,[a] 1 h after challenge with *P. gingivalis* cells

Immunization profile	Cytokine (pg/ml)			Outcome after 14 days
	IFN-γ	TNF-α	IL-4	
PgF-P8, 21 days	6,801	4,364	3,862	Died
	34,817	2,049	3,828	Died
	58,537	2,008	3,828	Died
	34,817	2,873	3,018	Died
	44,926	1,967	2,674	Died
	37,019	4,478	4,378	Died
	39,621	2,008	6,260	Died
	44,635	8,634	5,375	Died
	27,511	0	3,690	Died
	78,553	979	5,174	Died
Mean ± SD	40,724 ± 18,431	2,936 ± 2,422	4,209 ± 1,105	
PgF-P8, 40 days	6,309	5,715	6,933	Died
	11,335	7,562	5,142	Survived
	10,349	8,013	2,711	Died
	2,662	4,049	5,705	Died
	14,784	7,607	7,100	Died
	14,390	7,652	2,777	Survived
Mean ± SD	9,972 ± 4,723	6,766 ± 1,560	5,078 ± 1,916	
BSA, 21 days	99,871	4,848	3,406	Died
	39,021	2,173	2,772	Died
Mean ± SD	69,449 ± 43,027	3,511 ± 1,892	3,089 ± 448	
BSA, 40 days	10,152	6,481	3,076	Died
	9,068	6,166	4,928	Died
	19,515	7,472	5,601	Survived
	55,980	6,031	5,366	Died
	119,646	5,625	4,354	Died
	30,159	5,490	8,013	Died
Mean ± SD	40,753 ± 42,331	6,211 ± 716	5,223 ± 1,637	
Nonimmunized controls	33,716	3,078	3,258	Died
	28,512	938	4,418	Survived
	56,135	979	3,921	Died
	39,922	3,984	3,283	Died
Mean ± SD	39,571 ± 11,987	2,245 ± 1,531	3,720 ± 557	

[a] Cytokine levels of representative individuals of each group.

served a peak of response 24 to 48 h after the challenge. These curves showed no correlation with the outcome of the infection and the quantity, the kinetics, or the type of cytokine produced. Although Fig. 4 shows only three individuals per group, no clearer pattern emerged when larger groups of animals were tested. Likewise, we could not find any correlation either with survival and IL-2 or IL-6 levels at any time point (data not shown). Animals sham challenged with Wilkins-Chalgren broth did not show any cytokine elevation (data not shown).

DISCUSSION

Using a truncated form of the *P. gingivalis* fimbrillin, PgF1-198, we were able to identify a portion of the N terminus of the molecule containing T-cell epitopes. Although we were not able to obtain SI as high as the one obtained with the whole protein, PgF1-198 induced proliferation of the primed T cells. One of the reasons that could explain the lower proliferation is the high tendency of the truncated protein to aggregate in RPMI medium, probably because of the pH. Effectively, we found that refolding of the protein after denaturation required that the pH be maintained above 9.0. Significant aggregation was observed when the protein was diluted in culture medium (pH 7.3). The aggregation undoubtedly led to a decrease in the amount of protein presented to the T cells.

To further map the epitopes, we used synthetic peptides of 20 aa corresponding to this reactive portion of the molecule to restimulate r-fimbrillin-primed T cells. This allowed us to identify a very immunogenic sequence of the fimbrillin that corresponds to aa 103 to 122 of the native form. In fact, this peptide, PgF-P8, was able to stimulate T cells at the same level as the whole protein, with an SI equal to 19. PgF-P8 induced production of both Th1 and Th2 cytokines in in vitro restimulation of r-fimbrillin-primed T cells, as demonstrated by ELISA of the growth supernatant. IFN-γ was detected in growth supernatant at very high concentrations, i.e., >93,000 pg/ml, 48 h after in vitro restimulation with PgF-P8 or r-fimbrillin. IL-2, another Th1 cytokine, along with IL-4, a Th2 cytokine, was also detected at levels of 6,202 and 28 pg/ml, respectively, in the supernatant of cells restimulated with PgF-P8. The levels of these two cytokines were lower in cells restimulated with r-fimbrillin. This cytokine pattern is in agreement with results recently reported by Ogawa et al. (21). Using peptides covering the entire protein sequence, these authors reported the presence of Th1 epitopes between aa 116 and 125 and of Th2 epitopes between aa 106 and 125, areas that are covered by PgF-P8.

PgF-P8 not only was reactive with T cells but also induced increased titers of specific antibodies in mice immunized with the whole molecule, the r-truncated protein PgF1-198, or the peptide PgF-P8. The peptide induced the highest antibody titers, followed by PgF1-198 and finally by the whole r-fimbrillin. Although the whole molecule did not induce very high titers, antibodies to PgF-P8 reacted strongly with the r-fimbrillin as well as with PgF1-198 (data not shown).

We next wanted to investigate whether these T-cell and B-cell reactivities of PgF-P8 could confer protection against *P. gingivalis* infection. The mouse chamber model (6) was chosen for this purpose. With this model, it was shown that immunization with heat-killed *P. gingivalis* cells effectively inhibited the invasion of the surrounding tissues after subsequent challenge with invasive strains of *P. gingivalis*, thus reducing the death rate of the mice (7). Although the dramatic outcome of this model (i.e., death of the infected animal) does not exactly reflect the events occurring in human periodontal diseases, it offers an excellent tool to expose bacterial cells to the host mechanisms of defense with the possibility of continuous access to the chamber content for sampling and analysis, allowing longitudinal studies of local reaction of the same individuals. We used strain ATCC 33277 for our infection experiments, even though our r-fimbrillin gene was isolated from strain 381 (26). It is well known that these two strains are closely related and that their fimbrillin genes are identical (5). In preliminary experiments using this system, we had already observed protection induced by immunization with either r-fimbrillin or PgF1-198 of a limited number of animals (data not shown).

Nonimmunized CBA/J mice challenged with 3×10^{10} *P. gingivalis* ATCC 33277 CFU had a death rate of 75%. We were able to protect them by immunization with PgF-P8 in the footpad prior to challenge with the same inoculum. But, in order to get protection, we had to wait 40 days after the immunization before challenging. Animals immunized for a shorter period of time, 21 to 27 days, were not protected and had death rates in the same range as did naive animals or those injected with BSA. Animals challenged 40 days after immunization had much lower death rates (40%), and the deaths

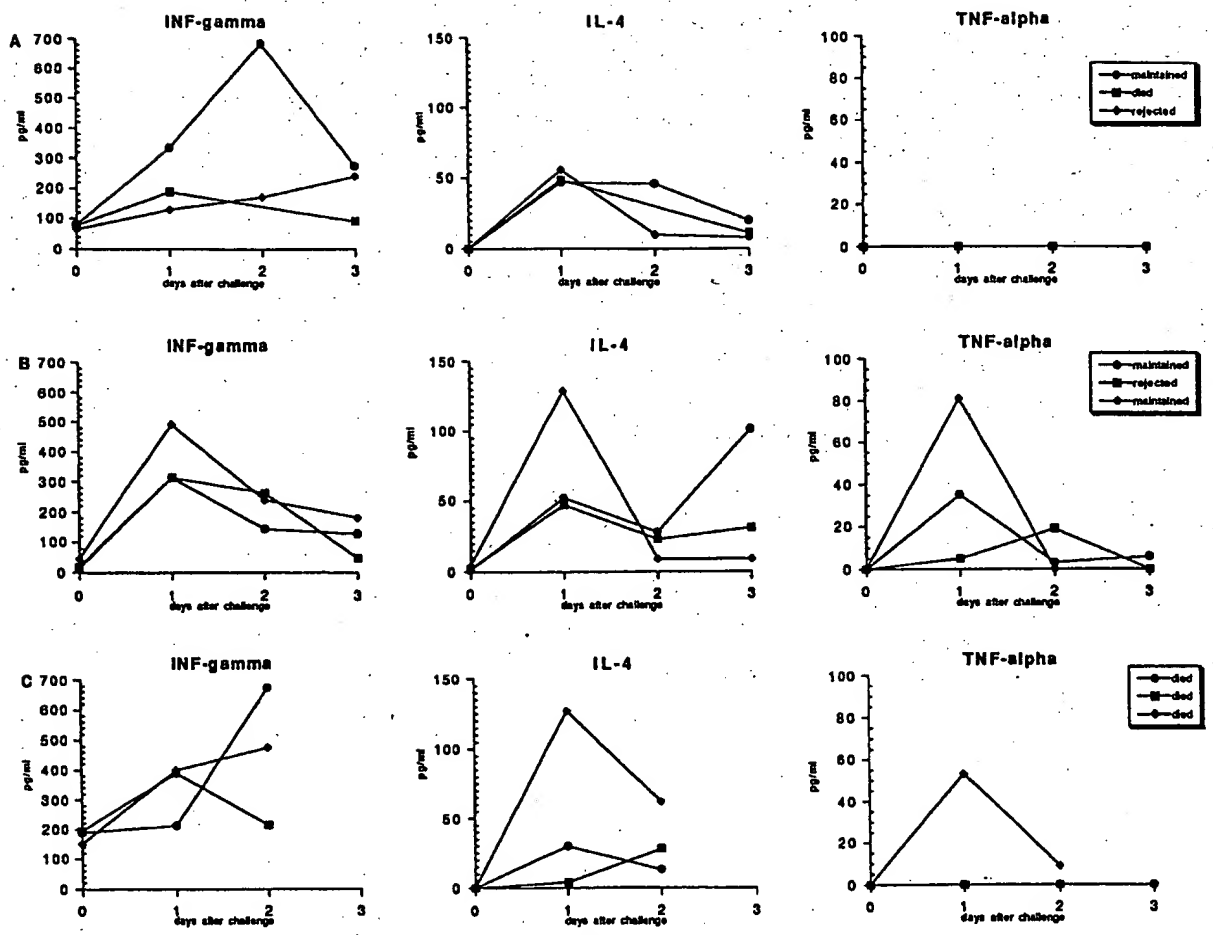

FIG. 4. Kinetics of cytokine production in the chamber after *P. gingivalis* challenge. CBA/J mice received 3×10^{10} *P. gingivalis* ATCC 33277 CFU in their chamber. Fluids were collected daily after the challenge and assayed by ELISA for IFN-γ, IL-4, and TNF-α levels. Three representative individuals from each group are shown here. (A) Nonimmunized controls ($n = 16$). (B) Mice immunized with PgF-P8 40 days prior to challenge ($n = 4$). (C) Mice immunized with PgF-P8 21 days prior to challenge ($n = 7$). The outcome of the infection for each individual animal appears on the right side.

occurred later after the challenge than for the ones challenged after 21 to 27 days or control groups.

The reason why animals immunized 21 days before challenge are not protected is unclear. One explanation could be that these animals still have high antibody titers to fimbrillin in the vicinity of the chamber that would cause immune complex formation upon challenge with bacterial cells. All immunized animals effectively had elevated specific serum antibodies, and these antibodies passed into the chamber, where they could be detected. However, there was no correlation between the antibody level and the outcome of the infection. Furthermore, even after 40 days postimmunization experimental mice still had relatively high titers of specific antibodies in the chamber. If immune complex formation were the reason for the lack of protection against *P. gingivalis* infection, mice immunized for 40 days would also not be protected. Furthermore, we did not see any correlation with the isotypes of antibodies to PgF-P8 present in the chamber and the infection outcome (data not shown). The most prevalent isotype of specific PgF-P8 antibodies, in both groups of immunized animals, was IgG1 (data not shown). The predominance of IgG1 antibodies in response to PgF-P8 is an agreement with earlier results on the nature of the antibody isotype produced against the whole fimbrillin in both mice (19) and humans (17).

Different cytokines produced during an immune response can exert striking and contrasting immunologic effects on the response to pathogens. The presence or absence of some cytokines can explain the progression or the elimination of an infection (4, 13, 22, 25). We thus analyzed the patterns of cytokines produced locally after the challenge with *P. gingivalis*. Immediately after the bacterial challenge, most animals showed elevated levels for the five cytokines tested, i.e., IFN-γ, TNF-α, IL-2, IL-4, and IL-6. However, the level of any individual cytokine failed to correlate with the outcome of the infection. IFN-γ and IL-2, both produced by Th1 cells, were not associated with any protection or susceptibility pattern. The same was true for the Th2 cytokine IL-4. TNF-α, mainly produced by activated macrophages, also failed to correlate with the infection outcome. This lack of correlation between immediate cytokine responses and the outcome of a bacterial challenge has been reported for other systems (1, 2, 25). Since most deaths occurred more than 48 h after the bacterial chal-

lenge, we did a longitudinal study of the cytokine responses to verify whether distinct kinetic patterns would be associated with protection and death. Again the mice showed an elevation of most cytokines after the bacterial challenge. That peak of response remained elevated for 24 to 48 h before dropping toward baseline levels. No kinetic patterns of cytokine production correlated with the outcome of the challenge. Therefore, we conclude that protection is not associated with preferred Th1 or Th2 cytokine patterns or TNF-α or IL-6 production.

Protection induced by immunization of rats with the whole fimbrillin protein (3) or hamsters with a synthetic peptide (15) has been previously reported. Here we identified a sequence of the fimbrillin molecule, corresponding to aa 103 to 122, responsible for secretion of both murine Th1 and Th2 cytokines and capable of dramatic protection against *P. gingivalis* infection in the mouse chamber model. Furthermore, this peptide contains amphipathicity A motifs and I-A^d and I-E^d binding motifs (15). Although most naturally occurring peptides eluted from class II molecules are 13 to 25 aa long (10, 24), some synthetic peptides as short as 11 aa still retain full antigenicity (23). Our peptide, a 20-mer, thus potentially contains more than one T-cell epitope. Whether one or several of these epitopes are responsible for the protection effect observed is still not known, and we need to study the protective capacity of shorter peptides covering the sequence of PgF-P8. Other T-cell protective epitopes might also be present in the other half of the molecule. In fact, we have already tested in vitro another r-truncated form of the fimbrillin ranging from aa 164 to 338 and found that it also induces T-cell proliferation. Peptide mapping of this portion is under way.

The protection induced by PgF-P8 was observed only when mice were challenged 40 days after immunization. Mice immunized for a lesser time were not protected. Although protection depended on the time of immunization before challenge, it did not correlate with cytokine production or specific antibody levels in vivo. We are currently investigating the nature of that protection.

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